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CUMENTATION PAGE

Form Approved OMB No. 0704-0188

(U)	1b. RESTRICTIVE MARKINGS: NA
2a. SECURITY CLASSIFICATION AUTHORITY: NA	3. DISTRIBUTION / AVAILABILITY OF REPORT: Distribution Unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE: NA	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): FAES	5. MONITORING ORGANIZATION REPORT NUMBER(S): NA
6a. NAME OF PERFORMING ORGANIZATION: FAES; 6b. OFFICE SYMBOL (If applicable): NA	7a. NAME OF MONITORING ORGANIZATION: Office of Naval Research
6c. ADDRESS (City, State, and ZIP Code): National Institutes of Health, Bethesda, MD 20892	7b. ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: Office of Naval Research; 8b. OFFICE SYMBOL (If applicable): ONR	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-87-G-0187
8c. ADDRESS (City, State, and ZIP Code): same as 7b	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
NO. 61153N	RR04108	441K701	

11. TITLE (Include Security Classification)
Surface and Molecular Forces Governing the Transport of Ions Across Electrically Excitable Membranes.

12. PERSONAL AUTHOR(S)
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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 5/1/87 TO 7/31/88	15th April 1988	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
ionic channels, surface potentials, ligand binding, voltage gating, surface energies, ion specificity, intermolecular forces, molecular assembly

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The aims of this project are:

to measure the internal aqueous volume changes during the gating of ionic channels by trans-membrane voltage;

to distinguish between various molecular structural models of channel gating;

to connect channel gating and specificity with measurements of intermolecular forces.

To this end, we have used osmotic stress to determine that a significant fraction of the internal aqueous volume is lost upon closure of two very different channels -- the anion channel from the outer membrane of mitochondria, and the potassium channel from the squid giant axon. The structural rearrangements that probably accompany such closure require that one recognize the likely motion of channel protein and the energetic importance of channel volume hydration. Protein motion immediately implicates mechanical properties of membrane lipids and proteins as part of the gating process; cavity hydration immediately requires recognition of the specific action of transversing ions on the energy of hydration.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	(U)

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
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DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

S/N 0102-LF-014-6603

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Introduction

Despite the enormous progress of past decades relating the passage of ionic currents across cell membranes to the electric fields that drive transport, despite recent advances delineating the properties of specific channels that open and close to enable ionic movement, despite molecular biological success synthesizing and modifying the proteins that make these channels, despite the major advances in the determination and representation of trans-membrane channel structures, we still have relatively weak ideas of how the applied fields couple with changes in protein structure to enable specific controlled transport. This project emphasizes two new approaches -- the measurement of forces that control protein structure and rearrangement and the modification of protein structure by osmotic stress applied to the aqueous cavities of proteins.

The major thrust of this work is to recreate the function of ionic channels in artificial bilayers or to observe channel behavior in natural cells and to subject them to osmotic stress. By this means we will measure the internal aqueous volume changes during gating by applied voltage. We will then distinguish between various structural models of channel gating. The rearrangements that accompany gating may be analyzed in terms of the electrochemical properties, particularly the ion-binding and the hydration properties, of the constituent macromolecular surfaces.

Methods

Proteins, especially those designed to transport ions across channels, are sensitive to osmotic stress from materials unable to enter their aqueous cavities. We have used the ability to exert osmotic stress to measure the amount of water that goes in or comes out when a protein, particularly a trans-membrane ionic channel opens or closes. Our method is a variant of the osmotic stress technique originally developed to measure forces between bilayer membranes and between macromolecules.

The experimental procedure is to measure the current voltage (I-V) relation for ionic channels, in intact cells or in reconstituted systems, and then to see how that relation is changed when the membrane channel protein is subjected to extra osmotic pressure. The shift in the I-V relation is a known function of (known) pressure x (unknown) volume change. With remarkable accuracy, then, one can determine the amount of water that must enter or leave a voltage-gated channel.

Requirements on the system are for good accuracy in recording electrical data and for good control of the osmotic conditions. It is enormously helpful to be able to observe single ionic channels and to be able to detect the discrete-step changes in conductance that signify single channel opening. To this end, much of our effort has been with the development of membrane reconstitution procedures and with the design and development of dedicated-computer hardware and software for ready analysis and control.

Results

During the past year we have performed measurements on K channels of the squid giant axon. We have determined that some 20 to 40 molecules of water enter the opening channel. Further, because of the selectivity of these channels, they are always under osmotic pressure from excluded solutes. In solutions of the tonicity normal for their function, these channels are unlikely ever to open 100% of the time even under optimal applied voltage. In fact, there seems to be a relatively weak link between the changes in structure that are associated with voltage gating and the actual steps of channel opening. A major problem with the procedure on intact axons is that one is looking at many channels and cannot distinguish a change in the probability of channel opening from a partial opening of any given channel. To this end, we are developing techniques for reconstituting single K channels to allow experiments analogous to those on the voltage-dependent anion channel (VDAC) to which the osmotic stress technique was first successfully applied.

We have succeeded in developing a simple procedure for solubilizing impure channel protein preparations, rapidly preparing vesicles with channels reconstituted therein, and separating vesicles containing large channels from those containing adsorbed protein or small channels. These vesicles can then be fused to planar bilayers with ease, since their osmotic properties are ascertained during the separation. We have used this procedure, originally calibrated with VDAC, for the reconstitution of Connexin32, the gap junction protein. We observe 20,50, and 150 pS channels in planar bilayers with slight anionic selectivity and assymetric voltage dependence.

Single channel work requires high temporal discrimination and control far sharper than what is the case on natural multi-channel preparations. We have been developing the use of dedicated personal computers as a laboratory tool for on-line data analysis. We choose to use personal based systems instead of remote multiuser mainframe computers for several reasons. First, our applications require a real time operating system and multi-user systems are by definition not real time controllers. Second, the rates required to transfer data to a remote mainframe are significantly beyond the capabilities of current network technology.

We are currently adapting a software and hardware system developed by Dr. F. Bezanilla (UCLA) for our single channel recording/data analysis. The system, which is based upon a personal computer, has both the time (11 microseconds per point) and voltage resolution (16-bit) sufficient for our applications. In addition, the system also has the capability of controlling the voltage across the channel by a high resolution (16-bit) digital to analog converter. This feature will allow the data recording to become more fully automated.

Finally, in anticipation of the importance of osmotic conditions on cellular activities other than trans-membrane transport, we have been studying the activity of aspartate transcarbamylase (ATCase) as a function of osmotic pressure. It is known from x-ray diffraction and centrifugation studies that the inactive (T) form of this enzyme has an aqueous cavity far smaller than that of the active (R) form. The hypothesis is that this

cavity will be subject to osmotic pressure from neighboring cellular components which can act to close down the protein. A new assay for the protein has been developed and a pHstat procedure for monitoring activity vs osmotic pressure has been set up, but there have been only preliminary results so far.

DISCUSSION AND PROSPECTS

Membrane transport is in large part due to membrane channels. Control of channel activity is the dominant theme in biological membrane selective permeability that underly activity in the nervous system, renal function, endocrine gland function, fluid secretion, and intracellular function. We think the detailed physical mechanisms of channel gating will illuminate both normal physiological and pathophysiological mechanisms of disease at a deeper level.

The structural rearrangements that appear to accompany channel closure require that one recognize the likely motion of channel protein and the energetic importance of channel volume hydration. Protein motion immediately implicates mechanical properties of membrane lipids and proteins as part of the gating process; cavity hydration immediately requires recognition of the specific action of traversing ions on the energy of opening.

We believe we are recognizing a previously neglected but essential variable in the control of channel and cellular activity. Our immediate studies will be on the voltage dependent anion channel (VDAC) that has been reconstituted and whose change in aqueous content has already been measured. We will be particularly interested in the mechanical act of gating as it relates to the mechanical properties of proteins and the mechanical stress that is a natural part of their incorporation into lipids.

The project might also undertake the study of other proteins such as hemoglobin and enzymes such as aspartate transcarbamylase that possess aqueous cavities whose volumes change when they "switch" between states of different ligand affinity or activity. We view channels as proteins with specialized aqueous cavities.

We also expect to investigate the changes in binding of ions in polyelectrolyte gels of known structure. By monitoring the hydration of these gels in the presence of different ions we are able to develop a new physical understanding of the molecular basis of ionic specificity, a specificity that seems to be changed by controlled changes in molecular arrangement. Hydration forces, known to dominate the interaction of macromolecules approaching contact, are also likely to be an important energetic factor in the stability of aqueous cavities. The dependence of these interactions on the identity of bound ions implicates in turn the importance of surface hydration to ion specificity. Cavity hydration immediately requires recognition of the specific effect on the energy of channel opening on the action of traversing ions.

PUBLICATIONS

J. Zimmerberg & V.A.Parsegian, Water Movement during Channel Opening & Closing, J. Bioenergetics & Biomembranes 19:351-358 (1987)

Two papers on vesicular reconstitution and one on the measured changes in squid axon potassium channels are near completion for submission.

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